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RECEPTOR FOR A BACILLUS THURINGIENSIS TOXIN

Ans A1
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Related Applications

The present application is a continuation-in-part of US Serial No 08/326,117, filed
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Technical Field

The invention relates to receptors that bind toxins from *Bacillus thuringiensis* and
thus to pesticides and pest resistance. More particularly, the invention concerns
recombinantly produced receptors that bind BT toxin and to their use in assays for
improved pesticides, as well as in mediation of cell and tissue destruction, dissociation,
20 dispersion, cell-to-cell association, and changes in morphology.

Background Art

It has long been recognized that the bacterium *Bacillus thuringiensis* (BT)
produces bactericidal proteins that are toxic to a limited range of insects, mostly in the
25 orders Lepidoptera, Coleoptera and Diptera. Advantage has been taken of these toxins in
controlling pests, mostly by applying bacteria to plants or transforming plants themselves
so that they generate the toxins by virtue of their transgenic character. The toxins

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themselves are glycoprotein products of the *cry* gene as described by Höfte, H. *et al. Microbiol Rev* (1989) 53:242. It has been established that the toxins function in the brush border of the insect midgut epithelial cells as described by Gill, S.S. *et al. Annu Rev Entomol* (1992) 37:615. Specific binding of BT toxins to midgut brush border membrane vesicles has been reported by Hofmann, C. *et al. Proc Natl Acad Sci USA* (1988) 85:7844; Van Rie, J. *et al. Eur J Biochem* (1989) 186:239; and Van Rie, J. *et al. Appl Environ Microbiol* (1990) 56:1378.

Presumably, the toxins generated by BT exert their effects by some kind of interaction with receptors in the midgut. The purification of a particular receptor from *Manduca sexta* was reported by the present inventors in an article by Vadlamudi, R.K. *et al. J Biol Chem* (1993) 268:12334. In this report, the receptor protein was isolated by immunoprecipitating toxin-binding protein complexes with toxin-specific antisera and separating the complexes by SDS-PAGE followed by electroelution. However, to date, there has been no structural information concerning any insect receptor which binds BT toxin, nor have, to applicants' knowledge, any genes encoding these receptors been recovered.

Disclosure of the Invention

The present invention is based, in part, on the isolation and characterization of a receptor that is bound by members of the BT-toxin family of insecticidal proteins, hereinafter the BT-R₁ protein. The present invention is further based on the isolation and characterization of a nucleic acid molecule that encodes the BT-toxin receptor, hereinafter BT-R₁ gene. Based on these observations, the present invention provides compositions and methods for use in identifying agents that bind to the BT-R₁ protein as a means for identifying insecticidal agent and for identifying other members of the BT-R₁ family of proteins.

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Brief Description of the Drawings

Sub B1
Figure 1 show the nucleotide sequence and deduced amino acid sequence of cDNA encoding the BT-R₁ protein from *M. sexta*.

Sub B2
5 Figure 2 (panels a and b) shows a comparison of amino acid sequences of cadherin motifs (BTRcad-1 to 11) in BT-R₁ to those of other cadherins.

Figure 3 shows a block diagram of the cadherin-like structure of BT-R₁.

Figure 4 shows the clone characterization of the BamHI-SacI fragment of BT-R₁.

LM is HindIII cut Lambda marker; UP is the uncut plasmid clone; NP is NsiI cut plasmid; XP is XhoI cut plasmid; BSP is BamHI and SacI cut plasmid showing the cloned fragment from BT-R₁; RM is mRNA size marker; and RT1 and RT2 are

Figure 5 illustrates the detection of protein expression from the plasmid containing the Bam-Sac fragment of BT-R₁ using ³⁵S-methionine as a tag. LCR is a luciferase control mRNA to show that the rabbit reticulocyte lysates are functional; RR1 and RR2 are expression products of the Bam-Sac fragment of BT-R₁ produced in rabbit reticulocytes from mRNA; LCT is a luciferase control plasmid to show that the transcription/translation kit is functional; and TT1 and TT2 are expression products of the Bam-Sac fragment of BT-R₁ produced in a transcription/translation kit.

Figure 6 shows a radio-blot of the Bam-Sac fragment of BT-R₁ with ¹²⁵I-labeled Cry1Ab. BBMV is the brush border membrane vesicles from the midgut of *M. sexta* containing the wild-type BT-R₁ receptor protein; RBK is a rabbit reticulocyte blank; RR1 and RR2 are the expression products of the Bam-Sac fragment of BT-R₁ produced in rabbit reticulocytes from mRNA; TBK is a transcription/translation kit blank; TT1 and TT2 are expression products of the Bam-Sac fragment of BT-R₁ produced in a transcription/translation kit. The arrows point to two of the bands.

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Figure 7 shows the presence of a BT-R₁ homologue in Pink Bollworm and European Corn Borer identified using toxin binding similar to that used to identify the original BT-R₁ clone.

Figure 8 shows the binding of Cry1Ab to fragments of the BT-R₁ protein.

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Modes of Carrying Out the Invention

I. General Description

The present invention is based, in part, on the isolation and characterization of a novel protein expressed in the midgut of *Manduca sexta* that binds to members of the BT-toxin family of proteins, hereinafter the BT-R₁ protein. The present invention specifically provides purified BT-R₁, the amino acid sequence of BT-R₁, as well as nucleotide sequences that encode BT-R₁. The BT-R₁ protein and nucleic acid molecules can serve as targets in identifying insecticidal agents.

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II. Specific Embodiments

A. BT-R₁ Protein

Prior to the present invention, although members of the BT-toxin family of protein were known, no one had identified the receptor that is bound by these toxin proteins. The present invention provides, in part, the amino acid sequences of a BT-toxin receptor that is expressed in the midgut of *Maduca sexta*.

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In one embodiment, the present invention provides the ability to isolate or produce a previously unknown protein by using known purification methods, the cloned nucleic acid molecules herein described or by synthesizing a protein having the amino acid sequence herein disclosed.

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As used herein, BT-R₁ refers to a protein that has the amino acid sequence of BT-R₁ provided in Figure 1, as well as allelic variants of the BT-R₁ sequence, and conservative substitutions mutants of the BT-R₁ sequence that have BT-R₁ activity.

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BT-R₁ is comprised of a single subunit, has a molecular weight of 210 kD, and has the amino acid sequence provided in Figure 1. A prediction of the structure of BT-R₁ is provided in Figure 3.

5 The BT-R₁ protein of the present invention includes the specifically identified and characterized variant herein described, as well as allelic variants, conservative substitution variants and homologues (Figure 7) that can be isolated/generated and characterized without undue experimentation following the methods outlined below. For the sake of convenience, all BT-R₁ proteins will be collectively referred to as the BT-R₁ proteins, the BT-R₁ proteins of the present invention or BT-R₁.

10 The term "BT-R₁" includes all naturally occurring allelic variants of the *Manduca sexta* BT-R₁ protein provided in Figure 1. In general, naturally occurring allelic variants of *Manduca sexta* BT-R₁ will share significant homology, at least 75 %, and generally at least 90%, to the BT-R₁ amino acid sequence provided in Seq. ID No:2. Allelic variants, though possessing a slightly different amino acid sequence than Seq. ID No:2, will be
15 expressed as a transmembrane protein in the digestive tract of an insect or other organism. Typically, allelic variants of the BT-R₁ protein will contain conservative amino acid substitutions from the BT-R₁ sequence herein described or will contain a substitution of an amino acid from a corresponding position in a BT-R₁ homologue (a BT-R₁ protein isolated from an organism other than *Manduca sexta*).

20 *See B3* One class of BT-R₁ allelic variants will be proteins that share a high degree of homology with at least a small region of the amino acid sequence provided in Seq. ID No: __, but may further contain a radical departure from the sequence, such as a non-conservative substitution, truncation, insertion or frame shift. Such alleles are termed mutant alleles of BT-R₁ and represent proteins that typically do not perform the same
25 biological functions as does the BT-R₁ variant of Seq. ID No:2.

The BT-R₁ proteins of the present invention are preferably in isolated form. As used herein, a protein is said to be isolated when physical, mechanical or chemical

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methods are employed to remove the BT-R₁ protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated BT-R₁ protein. The nature and degree of isolation will depend on the intended use.

5 The cloning of the BT-R₁ encoding nucleic acid molecule makes it possible to generate defined fragments of the BT-R₁ proteins of the present invention. As discussed below, fragments of BT-R₁ are particularly useful in: generating domain specific antibodies; identifying agents that bind to toxin binding domain on BT-R₁; identifying toxin-binding structures; identifying cellular factors that bind to BT-R₁; isolating
10 homologues or other allelic forms of BT-R₁; and studying the mode of action of BT-toxins.

Fragments of the BT-R₁ proteins can be generated using standard peptide synthesis technology and the amino acid sequence of *Manduca sexta* BT-R₁ disclosed herein. Alternatively, as illustrated in Example 5, recombinant methods can be used to
15 generate nucleic acid molecules that encode a fragment of the BT-R₁ protein. Fragments of the BT-R₁ protein subunits that contain particularly interesting structures can be identified using art-known methods such as by using an immunogenicity plot, Chou-Fasman plot, Garnier-Robson plot, Kyte-Doolittle plot, Eisenberg plot, Karplus-Schultz plot or Jameson-Wolf plot of the BT-R₁ protein. Fragments containing such residues are
20 particularly useful in generating domain specific anti-BT-R₁ antibodies or in identifying cellular factors that bind to BT-R₁. One particular fragment that is preferred for use in identifying insecticidal agents is a soluble fragment of BT-R₁ that can bind to a member of the BT family of toxins. In Example 5, a fragment of BT-R₁ that binds to a BT-toxin is disclosed.

25 As described below, members of the BT-R₁ family of proteins can be used for, but are not limited to: 1) a target to identify agents that bind to BT-R₁, 2) a target or bait to identify and isolate binding partners and cellular factors that bind to BT-R₁, 3) an assay

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target to identify BT-R₁ and other receptor-mediated activity, and 4) a marker of cells that express a member of the BT-R₁ family of proteins.

B. Anti-BT-R₁ Antibodies

5 The present invention further provides antibodies that bind BT-R₁. The most preferred antibodies will selectively bind to BT-R₁ and will not bind (or will only bind weakly) to non-BT-R₁ proteins. Anti- BT-R₁ antibodies that are especially contemplated include monoclonal and polyclonal antibodies as well as fragments containing the antigen binding domain and/or one or more complement determining regions (CDRs) of these
10 antibodies.

Antibodies are generally prepared by immunizing a suitable mammalian host using a BT-R₁ protein (synthetic or isolated), or fragment, in isolated or immunoconjugated form (Harlow, Antibodies, Cold Spring Harbor Press, NY (1989)). Regions of the BT-R₁ protein that show immunogenic structure can readily be identified
15 using art-known methods. Other important regions and domains can readily be identified using protein analytical and comparative methods known in the art, such as Chou-Fasman, Garnier-Robson, Kyte-Doolittle, Eisenberg, Karplus-Schultz or Jameson-Wolf analysis. Fragments containing these residues are particularly suited in generating specific classes of anti-BT-R₁ antibodies. Particularly useful fragments include, but are
20 not limited to, the BT-toxin binding domain of BT-R₁ identified in Example 5.

Methods for preparing a protein for use as an immunogen and for preparing immunogenic conjugates of a protein with a carrier such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation with reagents such as carbodiimide may be used; in other instances linking reagents like those
25 supplied by Pierce Chemical Co., Rockford, IL, may be effective.

Administration of a BT-R₁ immunogen is conducted generally by injection over a suitable time period in combination with a suitable adjuvant, as is generally understood in

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the art. During the immunization schedule, titers of antibodies can be taken to determine adequacy of antibody formation.

Although the polyclonal antisera produced in this way may be satisfactory for some applications, for many other applications, monoclonal antibody preparations are preferred. Immortalized cell lines which secrete a desired monoclonal antibody may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the BT-R₁ protein or BT-R₁ fragment. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', of F(ab')₂ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of the BT-R₁ protein can also be produced in the context of chimeric or CDR grafted antibodies of multiple species origin.

As described below, anti-BT-R₁ antibodies are useful as modulators of BT-R₁ activity, are useful in *in vitro* and *in vivo* antibody based assays methods for detecting BT-R₁ expression/activity, in generating toxin conjugates, for purifying homologues of *Manduca sexta* BT-R₁, in generating anti-ideotypic antibodies that mimic the BT-R₁ protein and in identifying competitive inhibitors of BT-toxin/BT-R₁ interactions.

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C. BT-R₁ Encoding Nucleic Acid Molecules

As described above, the present invention is based, in part, on isolating nucleic acid molecules from *Manduca sexta* that encode BT-R₁. Accordingly, the present invention further provides nucleic acid molecules that encode the BT-R₁ protein, as herein defined, preferably in isolated form. For convenience, all BT-R₁ encoding nucleic acid molecules will be referred to as BT-R₁ encoding nucleic acid molecules, the *BT-R₁* genes, or *BT-R₁*. The nucleotide sequence of the *Manduca sexta* nucleic acid molecule that encodes one allelic form of BT-R₁ is provided in Figure 1.

As used herein, a "nucleic acid molecule" is defined as an RNA or DNA molecule that encodes a peptide as defined above, or is complementary to a nucleic acid sequence encoding such peptides. Particularly preferred nucleic acid molecules will have a nucleotide sequence identical to or complementary to the *Manduca sexta* DNA sequences herein disclosed. Specifically contemplated are genomic DNA, cDNAs, synthetically prepared DNAs, and antisense molecules, as well as nucleic acids based on an alternative backbone or including alternative bases, whether derived from natural sources or synthesized. A skilled artisan can readily obtain these classes of nucleic acid molecules using the herein described *BT-R₁* sequences. However, such nucleic acid molecules, are defined further as being novel and unobvious over any prior art nucleic acid molecules encoding non-BT-R₁ proteins. For example, the *BT-R₁* sequences of the present invention specifically excludes previously identified nucleic acid molecules that share only partial homology to *BT-R₁*. Such excluded sequences include identified members of the cadherin family of proteins.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid molecules that encode polypeptides other than BT-R₁. A skilled artisan can readily employ nucleic acid isolation procedures to obtain an isolated BT-R₁ encoding nucleic acid molecule.

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The present invention further provides fragments of the BT-R₁ encoding nucleic acid molecules of the present invention. As used herein, a fragment of a BT-R₁ encoding nucleic acid molecule refers to a small portion of the entire *BT-R₁* sequence. The size of the fragment will be determined by its intended use. For example, if the fragment is chosen so as to encode the toxin binding domain of BT-R₁ identified in Example 5, then the fragment will need to be large enough to encode the toxin binding domain of the BT-R₁ protein. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming. Fragments of the *Manduca sexta* BT-R₁ gene that are particularly useful as selective hybridization probes or PCR primers can be readily identified from the entire *BT-R₁* sequence using art-known methods.

Another class of fragments of BT-R₁ encoding nucleic acid molecules are the expression control sequence found upstream and downstream from the BT-R₁ encoding region found in genomic clones of the *BT-R₁* gene. Specifically, tissue and developmental specific expression control elements can be identified as being 5' to the BT-R₁ encoding region found in genomic clones of the *BT-R₁* gene. Such expression control sequence are useful in generating expression vectors for expressing genes in the digestive tract of a transgenic organism. As described in more detail below, a skilled artisan can readily use the *BT-R₁* cDNA sequence herein described to isolate and identify genomic *BT-R₁* sequences and the expression control elements found in the *BT-R₁* gene.

Fragments of the BT-R₁ encoding nucleic acid molecules of the present invention (i.e., synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding BT-R₁ proteins, can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, *et al.*, *J Am Chem Soc* (1981) 103:3185-3191, or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various

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modular segments of the *BT-R₁* gene, followed by ligation of oligonucleotides to build the complete modified *BT-R₁* gene.

The *BT-R₁* encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. As
5 described above, such probes can be used to identify nucleic acid molecules encoding other allelic variants or homologues of the *BT-R₁* proteins and as described below, such probes can be used to identify the presence of a *BT-R₁* protein as a means for identifying cells that express a *BT-R₁* protein. A variety of such labels are known in the art and can readily be employed with the *BT-R₁* encoding molecules herein described. Suitable
10 labels include, but are not limited to, biotin, radiolabeled nucleotides, biotin, and the like. A skilled artisan can employ any of the art-known labels to obtain a labeled *BT-R₁* encoding nucleic acid molecule.

D. Isolation of Other *BT-R₁* Encoding Nucleic Acid Molecules

15 The identification of the *BT-R₁* protein from *Manduca sexta* and the corresponding encoding nucleic acid molecules, has made possible the identification of and isolation of:
1) *BT-R₁* proteins from organisms other than *Manduca sexta*, hereinafter referred to collectively as *BT-R₁* homologues, 2) other allelic and mutant forms of the *Manduca sexta* *BT-R₁* protein (described above), and 3) the corresponding genomic DNA that contains the
20 *BT-R₁* gene. The most preferred source of *BT-R₁* homologues are insects, the most preferred being members of the Lepidopteran, Coleopteran and Dipteran orders of insects. Evidence of the existence of *BT-R₁* homologues is provided in Figure 7.

Essentially, a skilled artisan can readily use the amino acid sequence of the *Manduca sexta* *BT-R₁* protein to generate antibody probes to screen expression libraries
25 prepared from cells and organisms. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified protein (as described above) or monoclonal antibodies can be used to probe an expression library, prepared from a target organism, to

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obtain the appropriate coding sequence for a BT-R₁ homologue. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructing an expression cassette using control sequences appropriate to the particular host used for expression of the enzyme.

5 Alternatively, a portion of the BT-R₁ encoding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the BT-R₁ family of proteins from organisms other than *Manduca sexta*, allelic variants of the *Manduca sexta* BT-R₁ protein herein described, and genomic sequence containing the *BT-R₁* gene. Oligomers containing approximately 18-20 nucleotides (encoding about a 6-7 amino acid
10 stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives.

 Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively amplify/clone a BT-R₁-encoding nucleic
15 acid molecule, or fragment thereof. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other BT-R₁ encoding nucleic acid molecules. Regions of the *Manduca sexta* BT-R₁ gene that are particularly well suited for use as a probe or as primers can be readily identified by one skilled in the art.

20 Non-*Manduca sexta* homologues of BT-R₁, naturally occurring allelic variants of the *Manduca sexta* BT-R₁ gene and genomic BT-R₁ sequences will share a high degree of homology to the *Manduca sexta* BT-R₁ sequence herein described. In general, such nucleic acid molecules will hybridize to the *Manduca sexta* BT-R₁ sequence under high stringency. Such sequences will typically contain at least 70% homology, preferably at
25 least 80%, most preferably at least 90% homology to the *Manduca sexta* BT-R₁ sequence of Seq. ID No:1.

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In general, nucleic acid molecules that encode homologues of the *Manduca sexta* BT-R₁ protein will hybridize to the *Manduca sexta* BT-R₁ sequence under stringent conditions. "Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015M NaCl/0.0015M sodium titrate/0.1% SDS at 50°C., or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C., with washes at 42°C. in 0.2 x SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal.

The presence of similar receptors in noninsect organisms as well as other insects besides those harboring BT-R₁ is supported by the sequence similarity of the BT-R₁ protein to that of the various members of the cadherin superfamily of proteins, which are membrane glycoproteins believed to mediate calcium-dependent cell aggregation and sorting. See, for example, Takeichi, M. *Science* (1991) 251:1451; and Takeichi, M. *N Rev Biochem* (1990) 59:237.

Included in this superfamily are desmoglein, desmocollins, the *Drosophila fat* tumor suppressor, *Manduca sexta* intestinal peptide transport protein and T-cadherin. All of these proteins share common extracellular motifs although their cytoplasmic domains differ. Goodwin, L. *et al. Biochem Biophys Res Commun* (1990) 173:1224; Holton, J.L. *et al. J Cell Sci* (1990) 97:239; Bestal, D.J. *J Cell Biol* (1992) 119:451; Mahoney, P.A. *et al. Cell* (1991) 853; Dantzig, A.H. *et al. Science* (1994) 264:430; and Sano, K. *et al. EMBO J* (1993) 12:2249. Inclusion of BT-R₁ in the cadherin superfamily is further supported by the report that EDTA decreases the binding of *CryIAb* toxin of BT to the

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210 kD receptor of *M. sexta* (Martinez-Ramirez, A.C. *et al. Biochim Biophys Res Commun* (1994) 201:782).

It is noted below that the amino acid sequence of BT-R₁ reveals that a calcium-binding motif is present. This is consistent with the possibility that cells having receptors to bind toxin may themselves survive although they render the tissues in which they are included permeable to solutes and thus effect disintegration of the tissue. Such a mechanism is proposed for the death of insects that ingest the toxin via the epithelial cells in their midgut by Knowles, B.H. *et al. Biochim Biophys Acta* (1987) 924:509. Such a mechanism is also supported in part by the results set forth in Example 4 hereinbelow which indicate that the effect of the toxin on embryonic 293 cells modified to express the receptor at their surface is reversible.

E. rDNA Molecules Containing a BT-R₁ Encoding Nucleic Acid Molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain a BT-R₁ encoding sequences as herein described, or a fragment thereof, such as a soluble fragment of BT-R₁ that contains the BT-toxin binding site. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in vitro*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al., Molecular Cloning* (1989). In the preferred rDNA molecules of the present invention, a BT-R₁ encoding DNA sequence that encodes a BT-R₁ protein or a fragment of BT-R₁, is operably linked to one or more expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which the BT-R₁ encoding sequence is operably linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the

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replication or insertion into the host chromosome, and preferably also expression, of the BT-R₁ encoding sequence included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, enhancers, transcription terminators and other regulatory elements. Preferably, an inducible promoter that is readily controlled, such as being responsive to a nutrient in the host cell's medium, is used. Further, for soluble fragments, it may be desirable to use secretion signals to direct the secretion of the BT-R₁ protein, or fragment, out of the cell.

In one embodiment, the vector containing a BT-R₁ encoding nucleic acid molecule will include a prokaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule intrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or viral promoter capable of directing the expression (transcription and translation) of the BT-R₁ encoding sequence in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories (Richmond, CA), pPL and pKK223 available from Pharmacia, Piscataway, NJ.

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Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to variant rDNA molecules that contain a BT-R₁ encoding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing
5 convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are PSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the
10 present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, i.e., the neomycin phosphotransferase (*neo*) gene. Southern *et al.*, *J Mol Anal Genet* (1982) 1:327-341. Alternatively, the selectable marker can be present on a separate plasmid, and the two
15 vectors are introduced by cotransfection of the host cell, and selected by culturing in the presence of the appropriate drug for the selectable marker.

F. Host Cells Containing an Exogenously Supplied BT-R₁ Encoding Nucleic Acid Molecule

20 The present invention further provides host cells transformed with a nucleic acid molecule that encodes a BT-R₁ protein of the present invention, either the entire BT-R₁ protein or a fragment thereof. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a BT-R₁ protein are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the
25 expression vector and expression of a *BT-R₁* gene. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, the most preferred being cells that do not naturally express a BT-R₁ protein.

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Any prokaryotic host can be used to express a BT-R₁-encoding rDNA molecule.
The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with an rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of
5 vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, *Proc Acad Sci USA* (1972) 69:2110; and Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

With regard to transformation of vertebrate cells with vectors containing rDNAs,
10 electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, *Virol* (1973) 52:456; Wigler *et al.*, *Proc Natl Acad Sci USA* (1979) 76:1373-76.

Successfully transformed cells, i.e., cells that contain an rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting
15 from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, *J Mol Biol* (1975) 98:503, or Berent *et al.*, *Biotech* (1985) 3:208 or the proteins produced from the cell assayed via an immunological method.

G. Production of a BT-R₁ Protein Using an rDNA Molecule

The present invention further provides methods for producing a BT-R₁ protein that uses one of the BT-R₁ encoding nucleic acid molecules herein described. In general terms, the production of a recombinant BT-R₁ protein typically involves the following steps.

25 First, a nucleic acid molecule is obtained that encodes a BT-R₁ protein or a fragment thereof, such as the nucleic acid molecule depicted in Figure 1. The BT-R₁ encoding nucleic acid molecule is then preferably placed in an operable linkage with suitable control

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sequences, as described above, to generate an expression unit containing the BT-R₁ encoding sequence. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the BT-R₁ protein. Optionally the BT-R₁ protein is isolated from the medium or from the cells;

5 recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in an appropriate host. The construction of expression vectors that are operable in a variety of
10 hosts is accomplished using an appropriate combination of replicons and control sequences. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily
15 adapt any host/expression system known in the art for use with BT-R₁ encoding sequences to produce a BT-R₁ protein.

H. Identification of Agents and Cellular Constituents that Bind to a BT-R₁ Protein

20 Another embodiment of the present invention provides methods for identifying agents and cellular constituents that bind to BT-R₁. Specifically, agents and cellular constituents that bind to BT-R₁ can be identified by: 1) the ability of the agent/constituent to bind to BT-R₁, 2) the ability to block BT-toxin binding to BT-R₁, and/or 3) the ability to kill BT-R₁ expressing cells. Activity assays for BT-R₁ activity and
25 binding and competitive assays using a BT-R₁ protein are suitable for use in high through-put screening methods, particularly using a soluble fragment of BT-R₁ that contains the BT-toxin binding domain, such as that disclosed in Example 5.

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In detail, in one embodiment, BT-R₁ is mixed with an agent or cellular extract. After mixing under conditions that allow association of BT-R₁ with the agent or component of the extract, the mixture is analyzed to determine if the agent/component bound to the BT-R₁. Binding agents/components are identified as being able to bind to the BT-R₁. Alternatively or consecutively, BT-R₁ activity can be directly assessed as a means for identifying agonists and antagonists of BT-R₁ activity.

Alternatively, targets that are bound by a BT-R₁ protein can be identified using a yeast two-hybrid system or using a binding-capture assay. In the yeast two hybrid system, an expression unit encoding a fusion protein made up of one subunit of a two subunit transcription factor and the BT-R₁ protein is introduced and expressed in a yeast cell. The cell is further modified to contain 1) an expression unit encoding a detectable marker whose expression requires the two subunit transcription factor for expression and 2) an expression unit that encodes a fusion protein made up of the second subunit of the transcription factor and a cloned segment of DNA. If the cloned segment of DNA encodes a protein that binds to the BT-R₁ protein, the expression results in the interaction of the BT-R₁ and the encoded protein. This brings the two subunits of the transcription factor into binding proximity, allowing reconstitution of the transcription factor. This results in the expression of the detectable marker. The yeast two hybrid system is particularly useful in screening a library of cDNA encoding segments for cellular binding partners of BT-R₁.

The BT-R₁ protein used in the above assays can be: an isolated and fully characterized protein, a fragment of a BT-R₁ protein (such as a soluble fragment containing the BT-toxin binding site), a cell that has been altered to express a BT-R₁ protein/fragment or a fraction of a cell that has been altered to express a BT-R₁ protein/fragment. Further, the BT-R₁ protein can be the entire BT-R₁ protein or a defined fragment of the BT-R₁ protein. It will be apparent to one of ordinary skill in the art that

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so long as the BT-R₁ protein or fragment can be assayed for agent binding, e.g., by a shift in molecular weight or activity, the present assay can be used.

The method used to identify whether an agent/cellular component binds to a BT-R₁ protein will be based primarily on the nature of the BT-R₁ protein used. For example, a gel retardation assay can be used to determine whether an agent binds to BT-R₁ or a fragment thereof. Alternatively, immunodetection and biochip technologies can be adopted for use with the BT-R₁ protein. A skilled artisan can readily employ numerous art-known techniques for determining whether a particular agent binds to a BT-R₁ protein.

Agents and cellular components can be further, or alternatively, tested for the ability to block the binding of a BT-toxin to a BT-R₁ protein/fragment. Alternatively, antibodies to the BT-toxin binding site or other agents that bind to the BT-toxin binding site on the BT-R₁ protein can be used in place of the BT-toxin.

Agents and cellular components can be further tested for the ability to modulate the activity of a BT-R₁ protein using a cell-free assay system or a cellular assay system. As the activities of the BT-R₁ protein become more defined, functional assays based on the identified activity can be employed.

As used herein, an agent is said to antagonize BT-R₁ activity when the agent reduces BT-R₁ activity. The preferred antagonist will selectively antagonize BT-R₁, not affecting any other cellular proteins. Further, the preferred antagonist will reduce BT-R₁ activity by more than 50%, more preferably by more than 90%, most preferably eliminating all BT-R₁ activity.

As used herein, an agent is said to agonize BT-R₁ activity when the agent increases BT-R₁ activity. The preferred agonist will selectively agonize BT-R₁, not affecting any other cellular proteins. Further, the preferred antagonist will increase BT-R₁ activity by more than 50%, more preferably by more than 90%, most preferably more than doubling BT-R₁ activity.

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Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences of the BT-R₁ protein or BT-toxin. An example of randomly selected agents is the use of a
5 chemical library or a peptide combinatorial library, or a growth broth of an organism or plant extract.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis that takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be
10 rationally selected or rationally designed by utilizing the peptide sequences that make up the BT-R₁ protein and BT-toxin. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to a fragment of a BT-R₁ protein or BT-toxin.

The agents tested in the methods of the present invention can be, as examples,
15 peptides, small molecules, and vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents used in the present screening method. One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of the BT-R₁ protein or BT-toxin. Small peptide agents can serve as competitive
20 inhibitors of BT-R₁ protein activity.

Peptide agents can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production
25 systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

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Another class of agents of the present invention are antibodies immunoreactive with critical positions of the BT-R₁ protein. As described above, antibodies are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the BT-R₁ protein intended to be targeted by the antibodies.

- 5 Critical regions particularly include the BT-toxin binding domain identified in Example 5. Such agents can be used in competitive binding studies to identify second generation BT-R₁ binding agents.

The cellular extracts tested in the methods of the present invention can be, as examples, aqueous extracts of cells or tissues, organic extracts of cells or tissues or
10 partially purified cellular fractions. A skilled artisan can readily recognize that there is no limit as to the source of the cellular extract used in the screening method of the present invention. The preferred source for isolating cellular binding partners of BT-R₁ are cells that express BT-R₁ or cells that are in close proximity to BT-R₁ expressing cells.

An outline of one screening method is as follows. Cells are modified by
15 transfection, retroviral infection, electroporation or other known means, to express a BT-R₁ protein and then cultured under conditions wherein the receptor protein is produced and displayed. If desired, the cells are then recovered from the culture for use in the assay, or the culture itself can be used *per se*.

In the assays, the modified cells are contacted with the candidate toxin and the
20 effect on metabolism or morphology is noted in the presence and absence of the candidate. The effect may be cytotoxic -- i.e., the cells may themselves exhibit one of the indices of cell death, such as reduced thymidine uptake, slower increase in optical density of the culture, reduced exclusion of vital dyes (e.g., trypan blue), increased release of viability markers such as chromium and rubidium, and the like. The differential response
25 between the toxin-treated cells and the cells absent the toxin is then noted. The strength of the toxin can be assessed by noting the strength of the response.

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These assays may be conducted directly as described above or competitively with known toxins. For example, one approach might be to measure the diminution in binding of labeled BT cry toxin in the presence and absence of the toxin candidate.

In addition to simply screening candidates, the screen can be used to devise
5 improved forms of toxins which are more specific or less specific to particular classes of insects as desired. The ability to determine binding affinity (K_a and K_d), dissociation and association rates, and cytotoxic effects of a candidate allows quick, accurate and reproducible screening techniques for a large number of toxins and other ligands under identical conditions which was not possible heretofore. Such information will facilitate
10 the selection of the most effective toxins and ligands for any given receptor obtained from any desired host cell.

Competition assays may also employ antibodies that are specifically immunoreactive with the receptor. Such antibodies can be prepared in the conventional manner by administering the purified receptor to a vertebrate animal, monitoring antibody
15 titers and recovering the antisera or the antibody-producing cells for immortalization, to obtain immortalized cells capable of secreting antibodies of the appropriate specificity. Techniques for obtaining immortalized B cells and for screening them for secretion of the desired antibody are now conventional in the art. The resulting monoclonal antibodies may be more effective than the polyclonal antisera as competition reagents; furthermore,
20 the availability of the immortalized cell line secreting the desired antibody assures uniformity of production of the same reagent over time. The information and the structural characteristics of toxins and ligands tested will permit a rational approach to designing more efficient toxins and ligands. Additionally, such assays will lead to a better understanding of the function and the structure/function relationship of both
25 toxin/ligand and BT- R_1 analogs. In turn, this will allow the development of highly effective toxins/ligands. Ligands include natural and modified toxins, antibodies (anti-

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receptor and antiidiotypic antibodies which mimic a portion of a toxin that binds to a receptor, and whatever small molecules bind the receptors.

I. Uses of Agents that Bind to a BT-R₁ Protein

5 As provided in the Background section, BT-R₁ is the target for the insecticidal activity of BT-toxins. Agents that bind a BT-R₁ protein can be used: 1) to kill BT-R₁ expressing cells, 2) to identify agents that block the interaction of a BT-toxin with BT-R₁ and 3) in methods for identifying cells that express BT-R₁.

10 The methods employed in using the BT-R₁ binding agents will be based primarily on the nature of the BT-R₁ binding agent and its intended use. For example, a BT-R₁ binding agent can be used to: deliver a conjugated toxin to a BT-R₁ expressing cell; modulate BT-R₁ activity; directly kill BT-R₁ expressing cells; or screen for and identify competitive binding agents. An agent that inhibits the activity of BT-R₁ can be used to directly inhibit the growth of BT-R₁ expressing cells. Further, identified cellular factors
15 that bind to BT-R₁ can, themselves, be used in binding/competitive assays to identify agonist and antagonists of BT-R₁.

J. Methods for Identifying the Presence of a BT-R₁ protein or gene

20 The present invention further provides methods for identifying cells, tissues or organisms expressing a BT-R₁ protein or a BT-R₁ gene. Such methods can be used to diagnose the presence of cells or an organism that expresses a BT-R₁ protein *in vivo* or *in vitro*. The methods of the present invention are particularly useful in the determining the presence of cells that are a target for BT-toxin activity or for identifying susceptibility of an organism to a BT-toxin or BT-toxin-like agent. Specifically, the presence of a BT-R₁
25 protein can be identified by determining whether a BT-R₁ protein, or nucleic acid encoding a BT-R₁ protein, is expressed in a cell, tissue or organism.

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A variety of immunological and molecular genetic techniques can be used to determine if a BT-R₁ protein is expressed/produced in a particular cell or sample. In general, an extract containing nucleic acid molecules or an extract containing proteins is prepared. The extract is then assayed to determine whether a BT-R₁ protein, or a BT-R₁ encoding nucleic acid molecule, is produced in the cell.

For example, to perform a diagnostic test based on nucleic acid molecules, a suitable nucleic acid sample is obtained and prepared using conventional techniques. DNA can be prepared, for example, simply by boiling a sample in SDS. The extracted nucleic acid can then be subjected to amplification, for example by using the polymerase chain reaction (PCR) according to standard procedures, such as a RT-PCR method, to selectively amplify a BT-R₁ encoding nucleic acid molecule or fragment thereof. The size or presence of a specific amplified fragment (typically following restriction endonuclease digestion) is then determined using gel electrophoresis or the nucleotide sequence of the fragment is determined (for example, see Weber and May *Am J Hum Genet* (1989) 44:388-339; Davies, J. *et al. Nature* (1994) 371:130-136)). The resulting size of the fragment or sequence is then compared to the known BT-R₁ proteins encoding sequences, for example via hybridization probe. Using this method, the presence of a BT-R₁ protein can be identified.

To perform a diagnostic test based on proteins, a suitable protein sample is obtained and prepared using conventional techniques. Protein samples can be prepared, for example, simply by mixing a sample with SDS followed by salt precipitation of a protein fraction. The extracted protein can then be analyzed to determine the presence of a BT-R₁ protein using known methods. For example, the presence of specific sized or charged variants of a protein can be identified using mobility in an electric field. Alternatively, antibodies can be used for detection purposes. A skilled artisan can readily adapt known protein analytical methods to determine if a sample contains a BT-R₁ protein.

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Alternatively, BT-R₁ protein or gene expression can also be used in methods to identify agents that decrease the level of expression of a *BT-R₁* gene. For example, cells or tissues expressing a BT-R₁ protein can be contacted with a test agent to determine the effects of the agent on BT-R₁ protein/gene expression. Agents that activate BT-R₁ protein/gene expression can be used as an agonist of BT-R₁ activity whereas agents that decrease BT-R₁ protein/gene expression can be used as an antagonist of BT-R₁ activity.

K. Methods to Sensitize Cells

The present invention further provides methods of sensitizing cells such that they become susceptible to killing with a BT-toxin, or a BT-toxin analog. Specifically, host cells transformed to express BT-R₁ receptor, or a homolog of the BT-R₁ receptor, become sensitive to the mode of action of BT-toxins. The binding of a BT-toxin to a BT-R₁ receptor expressed on the surface of the transformed cells results in induction of a cellular death and apoptosis of the cell expressing the BT-R₁ receptor. Accordingly, the BT-R₁ receptor is an appropriate candidate for use in transforming cells in which it is desirable to induce cell death.

There are numerous situations in which it is desirable to introduce the selected gene into a selected population of cells, thus bringing about cell death. One such example is in the therapeutic treatment of cancer cells. In using specifically targeted vectors for delivery of BT-R₁-encoding DNA molecules into a tumor cell, tumor cells within a patient can be engineered to express a BT-R₁ protein. Such cells then become susceptible to death upon treatment with a BT-toxin. Since BT-toxin is not normally toxic to mammalian cells, this method is particularly applicable to inducing cell death in particular cells in a mammalian host. Other situations where it may be desirable to stimulate cell death in particular cells or cell lines are in the treatment of autoimmune disorders and in the treatment of cells harboring pathogens, such as malaria or HIV agents.

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The choice of the actual steps employed to introduce a BT-R₁-encoding DNA molecule into a cell to render the cells susceptible to treatment with BT-toxin is based primarily on the cell type that is to be altered, the conditions under which the cell type will be altered, and the overall use envisioned. A skilled artisan can readily adapt art-
5 known methods for use with the BT-R₁-encoding DNA molecule of the present invention.

L. Animal Models and Gene Therapy

The *BT-R₁* gene and the BT-R₁ protein can also serve as a target for generating transgenic organisms in which the pattern of BT-R₁ expression has been altered. For
10 example, in one application, BT-R₁ deficient insects or insect cells can be generated using standard knock-out procedures to inactivate a *BT-R₁* gene, or, if such animals are non-viable, inducible *BT-R₁* antisense molecules can be used to regulate *BT-R₁* activity/expression. Alternatively, cells or an organism can be altered so as to contain a *Manduca sexta* BT-R₁ encoding nucleic acid molecule or an antisense-BT-R₁ expression
15 unit that directs the expression of a BT-R₁ protein or an antisense molecule in a tissue specific fashion. In such uses, an organism or cells, for example insects or insect cells, is generated in which the expression of a *BT-R₁* gene is altered by inactivation or activation and/or replaced by a *Manduca sexta* *BT-R₁* gene. This can be accomplished using a variety of art-known procedures such as targeted recombination. Once generated, the
20 *BT-R₁* expression altered cells or organisms can be used to 1) identify biological and pathological processes mediated by the BT-R₁ protein, 2) identify proteins and other genes that interact with the BT-R₁ protein, 3) identify agents that can be exogenously supplied to overcome a BT-R₁ protein deficiency and 4) serve as an appropriate screen for identifying mutations within the *BT-R₁* gene that increases or decreases activity.

25 For example, it is possible to generate transgenic insects, such as members of the dipteran order, expressing the *Manduca sexta* minigene encoding BT-R₁ in a tissue

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specific-fashion and test the effect of over-expression of the protein in tissues and cells that normally do not contain the BT-R₁ protein.

M. Use of Expression Control Elements of the BT-R₁ Gene

5 The present invention further provides the expression control sequences found 5' of the of the newly identified *BT-R₁* gene in a form that can be used in generating expression vectors. Specifically, the *BT-R₁* expression control elements, such as the BT-R₁ promoter, that can readily be identified as being 5' from the ATG start codon in the *BT-R₁* gene, can be used to direct the expression of an operably linked protein
10 encoding DNA sequence. Since BT-R₁ expression is mostly tissue-specific, the expression control elements are particularly useful in directing the expression of an introduced transgene in a tissue specific fashion. A skilled artisan can readily use the BT-R₁ gene promoter and other regulatory elements to generate expression vectors using methods known in the art.

15 Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the
20 disclosure.

Example 1

Purification and Sequence Determination of BT-R₁ Protein

Midguts of *M. sexta* were extracted and the BT-R₁ protein purified according to
25 the method of Vadlamudi, R.K. *et al. J Biol Chem* (1993) 268:1233, referenced above and incorporated herein by reference. The electroeluted band was confirmed to contain BT-R₁ protein by binding to ¹²⁵I-*cryIAb* toxin. In gel electrophoresis, the protein bound

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to toxin had an apparent weight of approximately 210 kD under reducing and nonreducing conditions.

See B4
The purified electroeluted BT-R₁ was subjected to cyanogen bromide digestion and the cyanogen bromide fragments separated on a 17% high-resolution tricine SDS-polyacrylamide gel as described by Schagger, H. *et al. Anal Biochem* (1987) 166:368. The separated fragments were transferred to Problott membranes (Applied Biosystems) and five bands were extracted and subjected to microsequencing using standard instrumentation. The amino acid sequences obtained were:

1. (Met)-Leu-Asp-Tyr-Glu-Val-Pro-Glu-Phe-Gln-Ser-Ile-Thr-Ile-Arg-Val-Val-Ala-Thr-Asp-Asn-Asn-Asp-Thr-Arg-His-Val-Gly-Val-Ala;
2. (Met)-X-Glu-Thr-Tyr-Glu-Leu-Ile-Ile-His-Pro-Phe-Asn-Tyr-Tyr-Ala;
3. (Met)-X-X-X-His-Gln-Leu-Pro-Leu-Ala-Gln-Asp-Ile-Lys-Asn-His;
4. (Met)-Phe/Pro-Asn/Ile-Val-Arg/Tyr-Val-Asp-Ile/Gly;
5. (Met)-Asn-Phe-Phe/His-Ser-Val-Asn-Arg/Asp-Glu.

Example 2

Recovery of cDNA

An *M. sexta* cDNA library was constructed from midgut tissue in λ gt10 using the Superscript Choice System according to the manufacturer's instructions (Life Technologies, Inc.). Degenerate oligonucleotide probes were constructed based on the peptide sequences determined in Example 1 using the methods and approach described in Zhang, S. *et al. Gene* (1991) 105:61. Synthetic oligonucleotides corresponding to peptides 1-3 of Example 1 were labeled with $\alpha^{32}\text{P}$ using polynucleotide kinase and used as probes as described in the standard cloning manual of Maniatis, T. *et al. Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2nd ed. 1989). A clone hybridizing to all three probes identified from 40 positive clones as hybridizing to all three of the probes was plaque-purified from a screen

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of 4×10^5 recombinants and subcloned into pBluescript (Stratagene). It contained an insert of 5571 bp.

Double-stranded cDNA in pBluescript was sequenced in both directions by the dideoxy termination method with Sequanase (USB) according to the manufacturer's
5 instructions. The sequencing showed an open reading frame of 4584 base pairs or 1528 amino acids along with a polyadenylation signal at position 5561. The sequence obtained and the deduced amino acid sequence is shown in Figure 1.

Thus, the deduced protein has a molecular mass of 172 kD and a pI of approximately 4.5. The amino acid sequences of the cyanogen bromide fragments of
10 native receptor match perfectly within the deduced amino acid sequence. The open reading frame begins with an ATG that is flanked by the consensus translation initiation sequence GAGATGG for eucaryotic mRNAs as described by Kozak, M. *Nucleic Acids Res* (1987) 15:8125.

Sup 5 As shown in Figure 1, the deduced amino acid sequence includes a putative
15 signal, shown underlined, preceding the mature N-terminus Asn-Glu-Arg-etc. Eleven repeats (cad1-cad11) are shown in the extracellular region upstream of the membrane domain, shown with the heavy underline, at positions 1406-1427. The end of the 11th repeat is shown with an arrowhead. The positions of the five CNBR fragments are also shown under the complete sequence.

Sup 6 Figure 2 compares the BT-R₁ sequence obtained herein with other members of the
20 cadherin family. Like known cadherins, the external domain of BT-R₁ is highly repetitive and contains 11 repeats (cad1-cad11; see Figure 2 A). The other cadherins compared in Figure 2 B are mouse P cadherin (mP EC1); *Drosophila fat* EC18 (fat EC18) and protocadherin (PC42 EC2), and *Manduca sexta* intestinal transporter (HPT-1-EC-1).
25 The eleven repeats of the cadherin motif in BT-R₁ (cad1-cad11) are individually aligned with a single motif sequence from each of the other members of the cadherin family. Conserved residues are boxed. The greatest similarity of BT-R₁ to the cadherins is with

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the extracellular repeats of the cadherin motif of mouse P-cadherin, *Drosophila fat* tumor suppressor and the protocadherins, although homologies are not high (20-40 homology and 30-60 percent similarity). The conserved repeats of BT-R₁ included AXDXD, DXE, DXNDXXP, one glutamic acid residue and two glycine residues (Figure 2 B). Motifs
5 A/VXDXD, DXNDN are the consensus sequences for calcium binding and two such regions are present in a typical cadherin repeat. In all repeats of BT-R₁, the sequence DXNDN is preceded by 8 to 14 hydrophobic amino acids. Similar hydrophobic sequences also have been observed in the cadherins. The length of the hydrophobic stretches suggests that these areas are not transmembrane regions but that they represent J-
10 sheet structures commonly present in cadherin-like repeats. BT-R₁ contains a putative cytoplasmic domain of 101 amino acids, smaller than vertebrate cadherin cytoplasmic domains (160 amino acids), and shows no homology to any of the cadherin cytoplasmic domains or to cytoplasmic domains of other proteins to which it has been compared in a current sequence data base.

15 To confirm that the sequenced clone encoded full-length BT-R₁ protein, total mRNA was prepared from midguts of *M. sexta* subjected to Northern blot by hybridization with the antisense 4.8 kb SacI fragment of the BT-R₁ cDNA clone. The Northern blot analysis was conducted by hybridizing to the antisense probe at 42°C and 50% formamide, 5 X Denhardt's Reagent, 5 X SSCP and 50 µg/ml salmon sperm DNA.
20 The filter was then washed two times with 1 X SSC + 0.1% SDS and two times with 0.15 X SSC + 0.1% SDS at 42°C. Each wash was roughly 20 minutes. The filter was then exposed to X-ray film for 24 hours. The 4.8 kb probe hybridized to a single 5.6 kb band.

The BT-R₁ clone was translated using rabbit reticulolysate and the resulting
25 translated products were immunoprecipitated with antisera raised against native protein encoded by BT-R₁. For the *in vitro* translation, pBluescript plasmid containing BT-R₁ cDNA was linearized and transcribed with T₃ polymerase (Pharmacia). The translation

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was conducted according to manufacturer's instructions with nuclease-treated rabbit reticulolysate (Life Technologies, Inc.). After one hour of incubation at 30°C, the reaction mixture was combined with an equal volume of SDS buffer or lysed with 50 mM Tris buffer containing 1% NP40 and 250 mM NaCl (pH 8.0) for immunoprecipitation.

- 5 Preimmune serum was used as a control. Translation and immunoprecipitation products were electrophoresed on a 7.5% SDS-polyacrylamide gel fixed, treated with Enhance (Dupont NEN), dried and exposed to X-ray film for 12 hours.

Two protein bands of approximately 172 kD and 150 kD as determined by SDS-PAGE were obtained; it is postulated that the 150 kD translation product was due to
10 initiation of translation from an internal methionine at amino acid 242. This is consistent with the observations of Kozak, M. *Mol Cell Biol* (1989) 9:5073.

Thus, both results confirm that a full-length clone was obtained.

Example 3

15 Recombinant Production and Characteristics of the BT-R₁ Protein

The BT-R₁ cDNA clone was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and the construct transfected into COS-7 cells. Membranes isolated from the COS-7 transfectants were solubilized, electrophoresed and ligand blotted with ¹²⁵I-CryIAb toxin. The cells were harvested 60 hours after transfection,
20 washed with phosphate-buffered saline and lysed by freezing in liquid nitrogen. Cell membranes were prepared by differential centrifugation as described by Elshourbagy, N.A. *et al. J Biol Chem* (1993) 266:3873. Control cells were COS-7 cells transfected with pcDNA3.

The cell membranes (10 µg) were separated on 7.5% SDS-PAGE blotted to a
25 nylon membrane and blocked with Tris-buffered saline containing 5% nonfat dry milk powder, 5% glycerol and 1% Tween-20. The nylon membrane was then incubated with ¹²⁵I-CryIAb toxin (2 X 10⁵ cpm/ml) for two hours with blocking buffer, dried and

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exposed to X-ray film at -70°C . The labeled toxin bound to a 210 ± 5 kD protein; the 210 kD band was observed only in lanes containing membranes prepared from either *M. sexta* or COS-7 cells transfected with the BT-R₁ cDNA construct containing 4810 bp of cDNA comprising the open reading frame.

5 The discrepancy between the 210 kD protein expressed and the calculated 172 kD molecular weight is due to glycosylation of the protein; *in vitro* translation of the cDNA clone, as described above, which does not result in glycosylation, does produce the 172 kD protein. To verify this, the COS-7 produced protein was subjected to digestion with N-glycosidase-F by first denaturing the purified protein by boiling in 1% SDS for 5
10 minutes followed by addition of NP-40 to a final concentration of 1% in the presence of 0.1% SDS, and then incubating the denatured protein in sodium phosphate buffer, pH 8.5 at 37°C with N-glycosidase-F for 10 hours. Controls were incubated under the same conditions without enzyme. Digestion products were separated on a 7.5% SDS-PAGE and stained with Coomassie brilliant blue. This glycosidase treatment reduced the
15 molecular weight of BT-R₁ protein from 210 to 190 kD; this indicates N-glycosylation at some of the 16 consensus N-glycosylation sites in the protein. Treatment of BT-R₁ with O-glycosidase and neuraminidase did not alter the mobility of the protein.

 In addition, embryonic 293 cells were transfected with the BT-R₁ cDNA clone in pcDNA3 and incubated with the labeled toxin (0.32 nM) in the presence of increasing
20 concentrations (0 to 10^{-6} M) of unlabeled toxin. Nonspecific binding was measured as bound radioactivity in the presence of 1 TM unlabeled toxin. A value for the dissociation constant (K_d) of 1015 pM was determined by Scatchard analysis; this is approximately the same value that was obtained for the natural receptor as described by Vadlamudi, R.K. *et al. J Biol Chem* (1993) (*supra*).

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Example 4

Physiological Effect of BT Toxin on Modified Embryonic 293 Cells

Both unmodified embryonic 293 cells, and 293 cells which have been modified to produce the BT-R₁ receptor as described in Example 3, when cultured *in vitro* form
5 adherent star-shaped clusters. When BT toxin (200 nM) is added to serum-free medium, the clusters round up and release from the plastic surfaces of the culture dish. This effect is also observed under known conditions of cytotoxicity for 293 cells. The foregoing effect is observed only when the cells are cultured in serum-free medium since the toxin binds to serum and would thus be ineffective under conditions where serum is present.

10 However, in the presence of anti-receptor antisera, this effect of BT toxin is blocked. Also, when serum is added back to a culture of modified E293 cells which has been treated in serum-free conditions with the toxin, the cells revert to their normal star-shaped adherent cluster shapes. This indicates that the effect of the toxin is reversible.

15 Example 5

Identification Of A Fragment Of BT-R₁ That Binds To A BT Toxin

To understand some of the properties of BT-R₁, research has been undertaken to define the location of the BT-R₁/Cry1Ab protein-protein interaction. The full-length wild-type amino acid sequence of BT-R₁ is provided in Fig. 1 with a block diagram of a
20 possible cadherin-like structure for BT-R₁ shown in Fig 3. In both figures, restriction digest sites from the cDNA are provided relative to the positions at which they would disrupt the amino acid coding sequence.

A small fragment lying between the BamHI and SacI restriction sites of wild-type BT-R₁ was cloned into the vector pCITE (Novagen). This vector contains
25 transcription/translation sequences designed for use in a rabbit reticulocyte lysate (RRL) system. The clone has been analyzed by restriction mapping and mRNA expression (Fig. 4). Lane UP shows the uncut plasmid and lanes NP and XP show restriction digests

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using NsiI and XhoI, respectively. NsiI is used because it has only one restriction site lying within the Bam-Sac fragment and does not cut anywhere within the pCITE vector. The BSP lane shows the restriction digest of the clone using BamHI and SacI. The digest releases the cloned fragment which separates at about 700 base pairs. The RT1
5 and RT2 lanes show mRNA transcription from the clone after linearization with XhoI. The mRNA separates at the expected 1350 base pairs.

Protein for analysis has been prepared from this clone in two ways. First, an RRL translation kit was employed to produce protein from the mRNA transcription reaction described above. Second, the plasmid was added directly to an RRL based transcription
10 and translation (TNT) coupled kit. Protein production was detected using ³⁵S-methionine as a tag (Fig. 5). The LCR lane shows production of luciferase protein from mRNA in an RRL kit and the LCT lane is luciferase protein from a plasmid containing the luciferase coding sequence translated in the TNT kit. Both are positive controls to demonstrate that the two translation kits are operational. The major bands for luciferase
15 translation are observed at 66 kDa. The lanes labeled as RR₁ and RR₂ show expression of the polypeptide sequence of the Bam-Sac fragment of BT-R₁ translated from mRNA in the RRL kit. The lanes TT1 and TT2 are translations from the pCITE plasmid containing the Bam-Sac fragment from the TNT kit. All four lanes possess a major band at 30 kDa which is the expected size of the Bam-Sac fragment with the addition of a coded antibody
20 tag called S-tag. S-tag is part of the multicloning site of pCITE.

The clone was then tested for its ability to bind the insecticidal toxin Cry1Ab. Polypeptide translation of the Bam-Sac fragment of BT-R₁ was carried out in duplicate as described above. The only change is that the ³⁵S-methionine tag was left out of the reaction mixtures to produce non-radiolabeled proteins. The proteins were separated by
25 SDS-PAGE, blotted to nitrocellulose and hybridized with ¹²⁵I-labeled Cry1Ab (Fig. 6). BBMV is wild-type BT-R₁ prepared from the midgut brush border membrane vesicles (BBMV) of *M. sexta*, and, is used as a positive control. RBK and TBK are RRL and

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TNT control reactions prepared without mRNA or plasmid present to determine whether proteins endogenous to either kit bind Cry1Ab. R₁ and RR2 are translations from the RRL kit and TT1 and TT2 are from the TNT kit. A single 30-kDa band appears in each of these lanes. Two are marked by arrows. These bands demonstrate that the Bam-Sac
5 fragment of BT-R₁ is capable of binding Cry1Ab insecticidal toxin.

To further understand the nature of this binding site, a set of truncation mutants of BT-R₁ was prepared through the use of restriction digests. The cDNA was digested at specific sites to remove increasingly larger portions of the C-terminus. The restriction enzymes used were NsiI, BamHI, NruI, ClaI, XhoI and StuI (Figs. 1 and 3). The
10 procedure involved linearizing the plasmid at each one of these sites and transcribing up to the truncation. The shortened mRNAs then were translated in an RRL kit blotted to nitrocellulose and hybridized with ¹²⁵I-labeled Cry1Ab. Translation of the wild-type BT-R₁ from the cDNA showed binding to a 172-kDa protein band, the expected size of wild-type BT-R₁. It also shows smaller bands that bind Cry1Ab although the nature of
15 these bands has not been determined. A blank made by preparing an RRL reaction mixture without any mRNA gave several bands below 66 kDa that show some type of binding of Cry1Ab to the reticulocytes. The specificity of this binding has not been determined. The truncation mutants created by NsiI, BamHI, NruI, ClaI, XhoI and StuI restriction digests did not show any binding to Cry1Ab except in the region where the
20 reticulocytes bind Cry1Ab. This data demonstrates that the removal of the last 100 amino acids from wild type BT-R₁ by NsiI restriction results in the loss of the ability of BT-R₁ to bind Cry1Ab. This localizes the toxin binding site on the BT-R₁ clone and provides a soluble fragment of the receptor that can be used in toxin and other binding studies.

25 A clone of a fragment of BT-R₁, called the Bam-Sac fragment, has been prepared. It was prepared using BamHI and SacI restriction digests (Fig. 1) and cloning of the resulting fragment into a vector called pCITE. The polypeptide sequence was translated

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and tested for binding to the insecticidal toxin Cry1Ab (Figure 8). The Bam-Sac fragment binds to Cry1Ab, providing first insight into the location of the Cry1Ab binding site within the BT-R₁ sequence. It lies in the last 234 C-terminal amino acids. This evidence is further supported by a set of truncation mutants that has been prepared.

- 5 Removal of the 100 most C-terminal amino acids from wild type BT-R₁ results in the loss of Cry1Ab binding. The C-terminal end of BT-R₁ is the location of the Cry1Ab binding site.

Example 6

10 Identification Of Homologue of BT-R₁ That Binds To A BT Toxin

Western blots of tissue extracts prepared from Pink bollworm and European corn borer were prepared and probed with labeled Cry1a (Figure 7). The results show that homologues of BT-R₁ are present in these two insects and can be readily isolated using the methods described herein.

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